INOCULATION OF *Pinus caribaea* WITH PURE CULTURES OF MYCORRHIZAL FUNGI IN PUERTO RICO

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INTRODUCTION

At the meeting of the IUFRO International Working Group on Mycorrhizae in Vienna in 1961, Hacskaylo (1962) reported that inoculation of soil with mycorrhizal fungi in Puerto Rico resulted in successful establishment of several species of pine. Many previous attempts to grow pine from seed met with total failure. Since 1961, we have followed the progress of introduction of pines on the island and have engaged in experimental work on responses of Caribbean pine (*Pinus caribaea*) to inoculations with pure cultures of mycorrhizal fungi. This report presents the historical background on the introduction of pine in Puerto Rico and some of the results of field and nursery inoculations trials that included pure cultures of known mycorrhizal fungi.

Historical Review

By reviewing correspondence and other documents in the files at the Institute of Tropical Forestry in Rio Piedras we were able to trace specific attempts to establish pine on the island as far back as 1930. In 1935, Gearhart wrote, "I recall when I arrived in Puerto Rico in October, 1928, that prior to that time numerous attempts were made in this nursery to propagate the Santo Domingan pine." There are no records of success except for a single tree of *Pinus occidentalis* planted on January 21, 1930 at the Utuado Nursery. On June 11, 1932, this tree was 1.09 m tall; on September 10, 1945, it was 9.3 m tall and 18.5 cm in diameter. The tree was destroyed for a building site in 1950. Gearhart listed the following species as those used in attempts to establish pine during 1929 and 1930: *P. attenuata*, *P. canariensis*, *P. caribaea*, *P. densiflora*, *P. insularis*, *P. maricata*, *P. pinaster*, *P. radiata*, and *P. torreyana*. He wrote, "All species germinated splendidly and grew 3 to 4 inches in a relatively short time, but succumbed after the 4th and 5th month."

In 1948, Dr. Frank Wadsworth, Director of the Institute of Tropical Forestry, inquired in correspondence with members of the Southern Forest Experiment Station whether or not mycorrhizae might be important in establishing pines. José Marrero noted that there had been correspondence with a soils specialist at Cornell University, as well as with the Southern Forest Experiment Station, about the question of the lack of mycorrhizae. Dr. Earl Stone, of Cornell University, in 1948 wrote that he believed applications of superphosphate would induce the seedlings to grow. Many combinations of superphosphate and other phosphates were applied but the results as recorded were total failures. In 1949, Gilormini suggested the possibility of transporting mycorrhizal inoculum in an "artificial propagating medium", but did not find such a medium.

In March, 1955, some members of the Forest Service from the Southern Forest Experiment Station visited Puerto Rico and considered the potential significance of inoculating pines with mycorrhizal fungi. In June, 1955, they collected soil samples of shortleaf (*Pinus echinata*) and loblolly (*P. taeda*) pines growing in a 25-year-old stand near Battle Creek, North Carolina. They used eight small cardboard containers, filling two with humus, and two with A_1 Horizon material from beneath each species of pine. The containers were then shipped to Puerto Rico. Using the soil inoculum one-year-old seedlings about a foot high and described as "scrawny", were inoculated by Forest Service personnel at Maricao in the western mountains of the island. There was a total of 64 seedlings in randomized plots. Thirty-two were inoculated, and 32 remained as uninoculated controls. Four seedlings each were used as replicates and the 8 collections of inoculum were used as treatments. Two-hundred cubic centimeters of inoculum were incorporated into the top inch and a half of soil in an area about 15 cm in diameter around the seedling. The soil around the control plants was dug and mixed similarly, but no inoculum was added.

By June, 1956, the roots of the inoculated seedlings had developed abundant mycorrhizae. Most of the controls had died, but those that remained had no mycorrhizae. The tallest of the inoculated seedlings was approximately 1.5 m in height.

Between 1956 and 1959, soil from beneath these trees was used as one source of inoculum for establishing mycorrhizae on pine seedlings in nurseries. The successful growth of the pines stimulated interest in their

silvicultural requirements for optimum growth in Puerto Rico. During the autumn of 1958, Throop, a research forester at the Institute, imported barerooted pine seedlings from Florida. Seedlings of *Pinus elliottii* and *P. taeda* were planted at several sites on the island to determine their adaptability. In February, 1959 Hacskaylo made an inspection tour of the island to examine the status of mycorrhizal development on the introduced pines and to evaluate the effects of inoculation trials. Careful examination of pines from ten different locations revealed that in general those with mycorrhizae were far superior to those without them. Upon returning to Beltsville, Maryland, Hacskaylo sent a mixture of duff containing mycorrhizal fungi. The results were consistently successful (Briscoe, 1959).

At the 1961 meeting the Mycorrhiza Working Group discussed the inoculation trials in Puerto Rico. Several members expressed the desire to hold a field meeting there and to inspect the results of these inoculations. Later it was decided that such a meeting would be held in July of 1964. In preparation we shipped pure cultures of four mycorrhiza fungi growing in Moser's (1958) peat moss medium to Puerto Rico in December, 1963. Also included were spores of three mycorrhizal fungi provided by Professor Melin of Uppsala, Sweden. Dr. C. B. Briscoe at the Institute of Tropical Forestry inoculated seedlings of *Pinus strobus, P. chiapensis, P. pseudostrobus,* and *P. caribaea* with the mycelia and spores, and planted them on six different sites around the island. There was insufficient time between field inoculations and the IUFRO Group inspection to make other than preliminary determinations. Unfortunately climatic conditions thereafter were severe and all of these young seedlings perished. As a result of this meeting and other contacts between certain working group members, it was decided that the study could profitably be repeated. In a special report, Professor Björkman (1964) made this as a specific recommendation. Thereafter, the U. S. Forest Service, with Hacskaylo and Vozzo as investigators in cooperation with the Institute of Tropical Forestry, embarked upon a two-year program to study the effects of inoculation of pines in Puerto Rico with mycorrhizal fungi. In addition we obtained support from the National Science Foundation (Grant No. GB 3978) through The George Washington University to subsidize travel expenses for Mr. Vozzo. Since April, 1965 he has made periodic visits of several days, approximately once a month. Materials were brought back to our laboratory at Beltsville for intensive study.

The following is a preliminary summary of the experimental work with some of the results. More specific details will be reported elsewhere.

EXPERIMENTAL

The inoculation trials were divided into 2 experiments. In the first, seedlings were outplanted from the nursery to a field site near Humacao. Heavy seedling losses caused by vandalism, grazing cattle, iguana egg deposits in seedling holes, and so forth, prompted the decision to retain the seedlings in the nursery at the Institute of Tropical Forestry in Rio Piedras during the second experiment. Similar basic procedures were used each time.

Preparation of Inoculum

Inoculum was prepared according to the general procedure of Moser (1958), where sterilized peatmoss moistened with a nutrient solution served as the substrate for culturing the mycorrhizal fungi. We modified the procedure by mixing the peatmoss with vermiculite in a 2:1 ratio and moistened the medium with a glucose-ammonium tartrate nutrient solution (Melin and Das 1954). Polypropylene cups were filled with the moistened peatmoss-vermiculite mixture, then fitted with tight heavy paper lids that had a small hole bored in the top. The hole was plugged with cotton. The entire assembly was autoclaved for 30 minutes at 121°C. After autoclaving the medium was pH 3.8.

Four known mycorrhizal fungi were selected for the inoculation trials because they grew readily; some had distinctive colors and together they represented a wide spectrum in the types of ectotrophic organisms. They were: *Cenococcum graniforme*, (black), *Corticium bicolor*, (yellow), *Rhizopogon roseolus*, and *Suillus cothurnatus*[1/] The culture of *R. roseolus* originally came from Professor E. Melin of Sweden, and *C. bicolor* from Professor P. Mikola of Finland. The fungi were first subcultured on Hagem's agar (Modess 1941), then on Hagem's solution. Thereafter, the mycelial mats were macerated with glass beads and the resulting suspensions were used to inoculate the previously prepared cups. After 16 weeks in our laboratory the inoculum was shipped by air to San Juan, Puerto Rico.

1/ Determined by Orson K. Miller

Nursery Preparations

The seedlings were established as follows: a 1:1 mixture of peatmoss and vermiculite was moistened with water, then sterilized with methyl bromide. The mixture was used to fill plastic bags approximately 8 cm in diameter and 15 cm long with perforations in the sides. One seed of *Pinus caribaea* was sown directly into each of the bags. If a seed did not germinate the bag was reseeded. The seedlings were maintained in the nursery for 4 months where they were watered and lightly fertilized. At the end of this time they were sampled and were found to be free of mycorrhizae.

Inoculations

The seedlings were divided into 14 groups of 18 each. This permitted 2 replications of the following 7 treatments:

Control (no inoculum, no fertilizer)
Fertilizer (9-10-5 NPK) only
Cenococcum graniforme mycelium
Corticium bicolor mycelium
Rhizopogon roseolus mycelium
Suillus cothurnatus mycelium
Natural inoculum (soil containing mycorrhizal fungi)

For the pure culture inoculations one cup of inoculum was used for each 2 seedlings. The plastic bags were split, the inoculum placed against the exposed roots, then the bag was closed and slipped into a 16-ounce paper cup. The natural inoculum was obtained by digging soil from around pines with established mycorrhizae. The soil was mixed well and a small handful was used for each seedling, as above. The latter method is the commonly used procedure in nurseries in Puerto Rico. The controls and uninoculated but fertilized seedlings were kept separate from those that were inoculated. The uninoculated but fertilized seedlings thereafter received applications of 9-10-5 (NPK) commercial fertilizer every three weeks. After 6 additional weeks in the nursery, in early November 1965, the seedlings were moved to a site near Humacao for outplanting.

Outplanting

The site selected for outplanting was situated on a sandy-loam, grassy, gentle slope. It previously had been used for grazing but was fenced off

for the experiment. Average yearly rainfall for the area is 90-100 inches.

The seedlings were planted in the field in 14 plots of 18 seedlings to a treatment. Each plot was 5 m x 5 m with an empty plot between to isolate those with seedlings. Arrangements were made with the Institute of Tropical Forestry to water and weed the area as necessary. Weeding was a big problem due to rapid growth of grass which can amount to 12 to 15 cm per week.

RESULTS OF THE FIELD STUDY

Ten seedlings were sampled from each treatment and measured periodically (Table 1). The average heights from the cotyledonary scar to shoot apex were determined. Where mycorrhizae had formed individual

Table 1. Average heights of *Pinus caribaea* seedlings in the field experiments near Humacao, Puerto Rico. Measurements were from cotyledonary scars to shoot apices.

Treatment	Age in weeks from germination		
	18 (inoc.)	28	40
	cm	cm	cm
Suillus cothurnatus	5.8	11.2	13.3
Rhizopogon roseolus	4.5	10.0	11.4
Cenococcum graniforme	6.0	8.3	15.2
Corticium bicolor	7.2	8.3	11.4
Natural inoculum	5.1	8.1	12.4
Fertilizer only	5.7	7.3	8.8
Control	6.9	8.7	8.8

seedling heights were consistently greater than where the inoculation was unsuccessful. (Fig. 1). Not all seedlings developed mycorrhizae from the pure culture inoculum, but they were approximately 75 percent successful. Frequency of success was about 95% with the natural inoculum. These seedlings, unaccountably, in general also were darker green and were thriftier in appearance, but not necessarily taller than other inoculated seedlings.

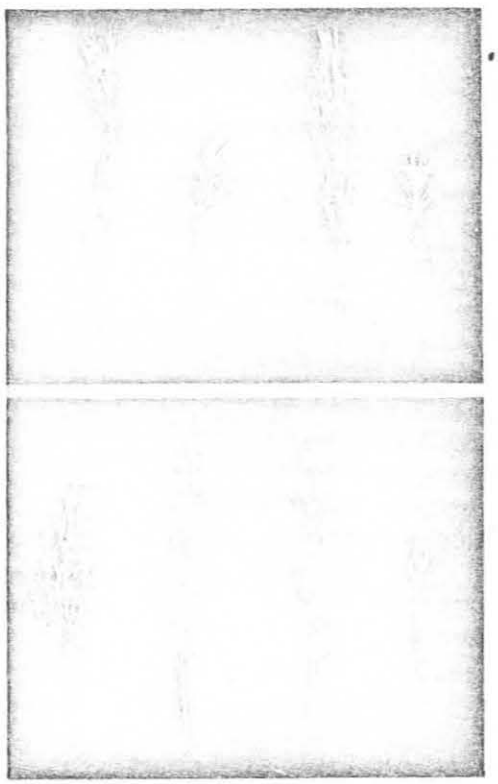

Fig. 1. *Pinus caribaea* seedlings inoculated with pure culture of *Suillus cothurnatus* (above) and natural inoculum (below). The large seedlings were mycorrhizal, the small ones were non-mycorrhizal.

The fertilized seedlings, like the controls, increased in height very little from the time they were transferred from the nursery to the field (Fig. 2).

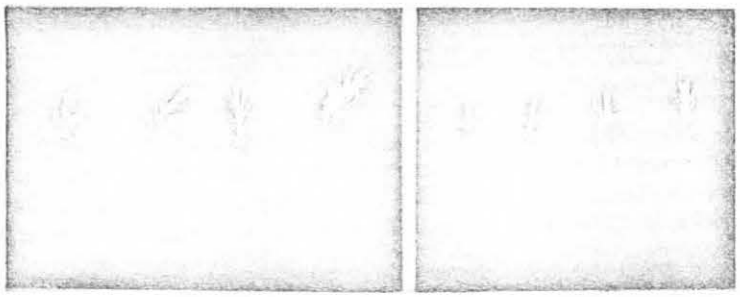

Fig. 2. *Pinus caribaea* seedlings uninoculated (left) and uninoculated but fertilized (right).

Very heavy losses were sustained as previously mentioned caused particularly by an invasion of grazing Brahma bulls that either ate or stepped on many of the seedlings. Consequently, the experiment was terminated after 6 months in the field. The last reliable seedling sample was at 22 weeks after inoculation. These results, however, were very striking when viewed as individual seedlings, and consequently we arranged to repeat the experiment in a nursery where the seedlings were safe from the perils encountered in the field.

RESULTS OF THE NURSERY STUDY

In May 1966, seeds of *Pinus caribaea* were germinated in sandy-loam soil that was sterilized with methyl bromide. After 18 weeks the seedlings were lifted, their roots examined, and found to be nonmycorrhizal. They were replanted in plastic bags of the sterilized soil. At the same time inoculum was added directly to the root systems as previously.

The basic design of the first experiment was replicated; however, the seedlings were retained in the Institute of Tropical Forestry nursery at Rio Piedras.

Seven treatments of 33 seedlings each were replicated 3 times for a total of 693 plants. The seedlings were watered three times each week or as needed. In contrast to the severe losses at Humacao survival of the seedlings was exceptionally good and general vigor was much better than in the field. Growth measurements began to show a differential within 10 weeks after inoculation (Table 2). The seedlings inoculated

Table 2. Average heights of *Pinus caribaea* seedlings in the nursery experiments in Río Piedras, Puerto Rico. Measurements were from cotyledonary scars to shoot apices.

Treatment	Age in weeks from germination					
	18 (inoc.)	23	28	34	38	43
	cm	cm	cm	cm	cm	cm
Suillus cothurnatus	7.9	8.6	11.9	20.6	25.9	32.0
Rhizopogon roseolus	7.7	8.6	12.6	22.2	26.8	34.9
Cenococcum graniforme	8.4	8.1	12.0	21.1	27.2	31.7
Corticium bicolor	8.5	9.9	17.3	30.3	39.3	44.8
Natural inoculum	7.9	9.0	11.1	18.2	23.4	32.3
Fertilizer only	7.4	8.6	10.1	15.0	25.4	29.3
Control	8.5	9.6	12.7	19.4	23.1	26.6

with *Corticium bicolor* were on the average larger than the others. This lead in height growth was sustained up to the time of this report. At 25 weeks after inoculation the other inoculated seedlings began to show increases over the controls. Those inoculated with natural inoculum were dark green in color as noted in the field experiment but so were most of the other inoculated seedlings. The fertilized seedlings were larger than the control seedlings, but were smaller than most of the inoculated ones. As noted in the field experiment the pure culture inoculations were about 75 percent effective. Seedlings that did develop mycorrhizae were consistently more vigorous and larger than nonmycorrhizal ones. These experiments are still in progress at this time and full details will be reported later.

SUMMARY AND CONCLUSIONS

Inoculation of seedlings of *Pinus caribaea* in Puerto Rico with pure cultures of 4 mycorrhizal fungi grown in a peatmoss-vermiculite medium resulted in formation of mycorrhizae on about 75 percent of the seedlings. Inoculations with soil containing mycorrhizal fungi was nearly always successful. In all cases, seedlings with mycorrhizae were more vigorous than nonmycorrhizal seedlings. Use of commercial fertilizer did not offset the stunting in seedlings that had no mycorrhizae. In the field experiments, use of soil inoculum resulted in darker green seedlings than in other treatments. In the nursery all inoculated seedlings that were mycorrhizal appeared dark green.

The favorable soil moisture conditions in the nursery apparently caused much better growth of all seedlings compared to the field experiment. Consequently, differences between treatments began to appear at a later seedling age. Other than *Corticium bicolor* in the nursery experiment our data did not show clearcut differences in inoculation with different species. These conclusions may be revised when additional data are available as the experiment progresses.

LITERATURE CITED

Björkman, E. 1964. Report and recommendations from IUFRO's Mycorrhiza Working Group (Section 24) meeting in Puerto Rico, July 2-7, 1964. Dept. of For. Bot., Royal Coll. of For. Pub. No. 137, 7 pp. Stockholm.

Briscoe, C. B. 1959. Early results of mycorrhizal inoculation of pine in Puerto Rico. Caribbean Forester 20 (3-4): 73-77.

Hacskaylo, E. 1962. Research on mycorrhizae in the United States. Proc. Int. Union of For. Res. Org., 13. Congress, Vienna 1961. 2:1,; section 24-6. 7 pp.

Melin, E. and V. S. Rama Das. 1954. Influence of root metabolites on the growth of tree mycorrhizal fungi. Physiol. Plant. 7: 851-858.

Modess, O. 1941. Zur Kenntnis der Mykorrhizabildner von Kiefer und Fichte. Symb. Bot. Ups. 5:1. 146 pp.

Moser, M. 1958. Die künstliche Mykorrhizaimpfung an Forstpflanzen. Forstw. Cbl. 77: 32-40.